ELSEVIER

International Journal of Pharmaceutics 144 (1996) 91–97

international journal of pharmaceutics

Degradation kinetics of growth hormone-releasing hexapeptide (GHRP-6) in aqueous solution

In Sik Ha[a], Byung Ho Woo[a], Jung Tae Lee[a], Tae Suk Kang[a], Kyung Kook Tak[a], Choon Kyung Oh[b], Kang Choon Lee[a,*]

[a] *Drug Targeting Laboratory, College of Pharmacy, SungKyunKwan University, 300 Chonchon-dong, Jangan-ku, Suwon City 440-746, South Korea*

[b] *Pharmaceutical Sciences, SmithKline Beecham Pharmaceutical, King of Prussia, PA 19406-0939, USA*

Received 3 July 1996; accepted 30 August 1996

Abstract

The degradation kinetics of growth hormone-releasing hexapeptide (GHRP-6) as a function of pH and temperature as well as buffer species and ionic strength has been studied by stability-indicating reversed-phase gradient high-performance liquid chromatography. Degradation chromatograms were confirmed in different pH solutions and the observed degradation reaction rates approximately followed first-order kinetics with respect to GHRP-6. The influence of the various buffer species (acetate, citrate, phosphate and borate) was shown to be different and the maximum stability of GHRP-6 was revealed to be in acetate buffer of pH 5.5–6.0. Degradation of GHRP-6 was greater in citrate-containing buffers than in acetate-containing ones. Furthermore, in the citrate-containing buffers, the higher buffer concentration caused greater degradation than the lower ones, but the concentration effect was negligible in acetate-containing buffers. Aqueous solution of GHRP-6 buffered with acetate (0.01 M, pH 5.5) showed a predicted $t_{90\%}$ of 4.73 years at 20°C.

Keywords: Peptide; Growth hormone-releasing hexapeptide; GHRP-6; Stability kinetics; Degradation; High-performance liquid chromatography

1. Introduction

GHRP-6 (His-D-Trp-Ala-Trp-D-Phe-Lys-NH_2) is a synthetic growth hormone-releasing hexapeptide which releases growth hormone in a dose-re-

* Corresponding author. Tel.: +82 331 2907704; fax: +82 331 2907724; e-mail: kclee@yurim.skku.ac.kr

PII S0378-5173(96)04719-9

lated, specific and non-species-specific manner, through mechanisms different from those of growth hormone-releasing hormone (Bowers et al., 1984, 1991; Bercu et al., 1991, 1992; Fletcher et al., 1994; Renner et al., 1994; Popovic et al., 1995; Fairhall et al., 1995).

Many aqueous formulations for parenteral, oral (Walker et al., 1990; Bowers et al., 1992; Hartman et al., 1992) and nasal administration (Nelson et al., 1991; Reardon et al., 1993; Vora et al., 1993) of GHRP-6 have been studied for use in clinical trials. It is confirmed that GHRP-6 is stable by leucine-aminopeptidase, α-chymotrypsin, human nasal perfusate and rabbit intestinal fluid (Oh et al., 1991). However, there is little fundamental information on the chemical and non-enzymatic stability of GHRP-6.

This study describes the degradation kinetics of GHRP-6 as a function of pH and temperature as well as buffer species and ionic strength by reversed-phase gradient high-performance liquid chromatography (HPLC).

2. Materials and methods

2.1. Materials

GHRP-6 was obtained from SmithKline Beecham Pharmaceutical (King of Prussia, PA) as the acetate salt. HPLC grade trifluoroacetic acid (TFA) and acetonitrile were obtained from Pierce and Fisher, respectively. All solutions and buffers were prepared with distilled deionized water that was filtered with a 0.22 μm membrane (Milli-Q system, Millipore) prior to use. All other materials were of reagent grade.

2.2. HPLC method

The reversed-phase HPLC system consisted of two Gilson 307 pumps, a Gilson 234 autoinjector, a 118 UV-Visible detector and a Gilson 712 system controller with 506C interface module (Gilson, Villiers-le-Bel, France). GHRP-6 and its degradation products were eluted from a Lichrosorb C-8 column (4.0 × 250 mm, 10 μm, Merck, Darmstadt, Germany) and detected at 215 nm. A linear gradient was employed; 20% A:80% B to 40% A:60% B over 20 min. Mobile phase A was 0.1% TFA/acetonitrile solution and mobile phase B was 0.1% TFA/distilled water. The injection volume was 10 μl and the flow rate was 1.2 ml/min.

A standard curve was constructed for each series of determinations over a range of 0.1–10 μg GHRP-6. The initial concentration of each GHRP-6 solution was designated 100%; all subsequent concentrations are expressed as a percentage of the initial concentration.

2.3. Kinetic studies

Test solutions were prepared with stock GHRP-6 solution of 1 mg/ml in Effendorff tubes as described below. The reaction tubes were then placed into constant-temperature water baths at the appropriate temperature of 40, 60, 80 and 90°C, respectively. Samples were periodically removed from the tubes with a Hamilton syringe and injected into the HPLC directly, as described previously (Lee et al., 1992).

Various pH buffer solutions with different species were prepared as follows: varying amount of 0.01 M sodium citrate and 0.02 M citric acid for pH 2.4–5.5 (citrate buffer); 0.01 M citric acid and 0.02 M dibasic sodium phosphate for pH 2.6–7.0 (citric/phosphate buffer); 0.01 M sodium acetate and 0.02 M acetic acid for pH 4.5–6.0 (acetate buffer); 0.01 M ammonium acetate/0.02 M acetic acid for pH 5.0–6.0 (ammonium acetate buffer); 0.01 M dibasic sodium phosphate and 0.02 M phosphoric acid for pH 5.5–8.0 (phosphate buffer); 0.01 M sodium borate and 0.02 M hydrochloric acid for pH 8.0–9.0 (borate buffer). The 10-times concentrated buffer solutions of the above were also prepared in the same manner. All buffer solutions were in the range 0.010–0.016 M or 0.10–0.16 M with regard to the mixture of corresponding buffer species.

Samples for ionic strength effect were prepared by adding 0.1 ml of GHRP-6 stock solution to vials containing 0.9 ml of respective buffer solution. To adjust the ionic strength of pH solution, potassium chloride was added to buffer solutions in suitable amounts to make solutions of 0.0–0.5 M potassium chloride.

3. Results and discussion

The correlation coefficient of the detector linearity for GHRP-6 in the concentration range 0.1–10 μg/ml was found to be greater than 0.999. The reproducibility at this concentration range was shown to be less than 0.5% S.D. ($n = 4$).

Fig. 1 illustrates the HPLC chromatograms of GHRP-6 kept in different pH solutions at 80°C. Several degradation products were detected and eluted after GHRP-6 in this HPLC system and appear to be relatively stable, since their concentration increases as the degradation of GHRP-6 progresses.

GHRP-6 contains five different amino acids including two ionizable groups of histidine and lysine. Therefore, GHRP-6 can exist in various ionic forms, depending on the pH of the aqueous solution, and all of them may have different tendencies to degradation in aqueous solution. In addition, two tryptophan and one phenylalanine in GHRP-6 are also amino acid groups susceptible to attack from certain environmental factors. Histidine may be racemized into D-His and also may be cyclized with neighboring tryptophan (Oyler et al., 1991). The side chains of tryptophan and histidine residues are also in general potential

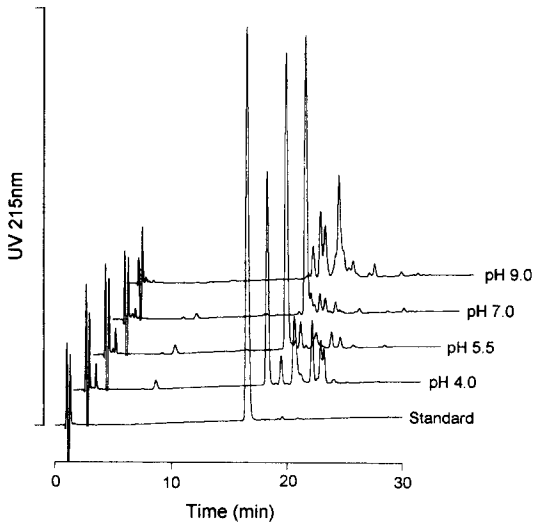

Fig. 1. HPLC chromatograms of GHRP-6 prepared in 0.01 M citric acid/0.02 M phosphate (pH 4.0–7.0) and 0.01 M HCl/0.02 M borate (pH 9.0) buffers at 80 ± 0.5°C for 72 h.

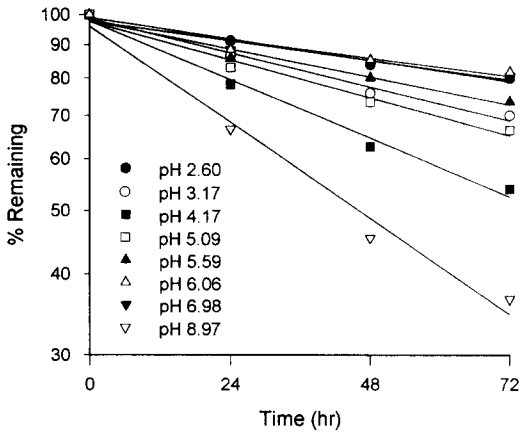

Fig. 2. First-order plot for the degradation of GHRP-6 in 0.01 M citric acid/0.02 M phosphate (pH 2.6–7.0) and 0.01 M HCl/0.02 M borate (pH 9.0) buffers at 80 ± 0.5°C.

oxidation sites (Manning et al., 1989; Samanen, 1991). Side chains of phenylalanine and lysine can be degraded via β-elimination at alkaline conditions (Manning et al., 1989). However, no attempt was made to determine the degradation products and specific degradation pathways for GHRP-6 in this study.

Fig. 2 shows a semilogarithmic plot of the residual percentage amount of GHRP-6 versus time in various pH solutions of 0.01 M citric acid and 0.02 M dibasic sodium phosphate at 80°C. It was found that the pH affected the degradation rate of GHRP-6 and that the observed degradation reaction rates approximately followed first-order kinetics.

The degradation of GHRP-6 was also conducted in aqueous solution from 40 to 90°C and at various pH of different buffer species. The observed reaction rate constants were obtained from the slopes of the semilog plots of concentration versus time by statistical regression analysis. The observed reaction first-order rate constants of GHRP-6 are listed in Table 1. The pH-rate profiles for the degradation of GHRP-6 were obtained by plotting the value of log k against pH as shown in Fig. 3.

The buffer solutions containing citrate ion showed remarkable differences in their degradation rate depending upon solution pH. Both cit-

Table 1
Observed rate constant for the degradation of GHRP-6

Buffer Solution	Temp. (°C)	pH	Rate constant (10^{-3}/h)
0.01 M citric acid/0.02 M phosphate	80	2.6	1.367
		3.2	2.179
		4.2	3.749
		5.1	2.445
		5.5	1.778
		5.8	1.299
		6.0	1.165
		7.0	1.249
0.01 M citrate/0.02 M citric acid	80	2.4	1.675
		3.3	4.172
		4.3	6.828
		5.5	2.176
0.01 M acetate/0.02 M acetic acid	80	4.5	1.083
		5.0	1.067
		5.5	0.972
		5.7	0.967
		6.0	0.975
0.01 M ammonium acetate/0.02 M acetic acid	80	5.0	1.056
		5.5	0.979
		6.0	0.951
0.01 M phosphate/0.02 M phosphoric acid	80	5.5	1.087
		6.2	1.126
		7.1	1.487
		8.0	2.784
0.01 M borate/0.02 M hydrochloric acid	80	8.0	2.016
		9.0	6.524
0.01 M sodium acetate/0.02 M acetic acid	40	5.5	0.012
	60	5.5	0.121
	80	5.5	0.972
	90	5.5	1.527

rate and citric/phosphate buffers showed similar pH profile and the data points connected by line segments illustrated the facing V-shaped curve of the profile in the pH range 2.6–7.0. But in acetate-containing buffers, GHRP-6 was proven to be extremely stable in the pH range 4.5–6.0, in which few differences in degradation reaction rate of GHRP-6 were observed. Although the exact pH optimum of GHRP-6 cannot be defined, GHRP-6 showed maximum stability pH ranges of 5.5–6.0. Accordingly, the degradation profiles of GHRP-6 in different buffers were compared at pH 5.5 and 80°C, as shown in Fig. 4. The highest stability of GHRP-6 was revealed to be in acetate buffer and, in contrast, buffers containing citrate ions caused greater degradation than acetate-containing buffers. This means that the stability of GHRP-6 in aqueous solution is strongly affected by the buffer species as well as by the solution pH.

The effect of buffer concentration on the degradation of GHRP-6 was examined at pH 5.5 and 80°C. As shown in Table 2, in the case of citrate-containing buffers, citrate and citric/phosphate buffers, the higher buffer concentrations caused greater degradation than the lower ones, apparently because of a catalytic effect of citrate. However, there was no remarkable difference between

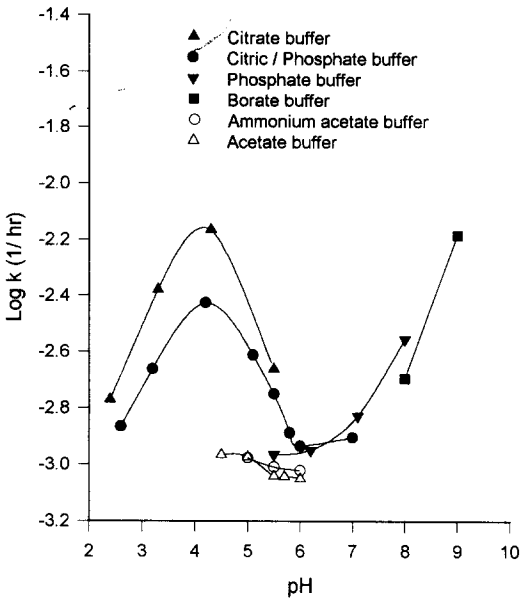

Fig. 3. The pH-rate profile of the degradation of GHRP-6 in different buffer solutions at 80 ± 0.5°C.

higher and lower concentrations of acetate-containing and phosphate buffers. Thus, acetate and phosphate ions have a smaller catalytic effect on the degradation of GHRP-6 in aqueous solution than do citrate ions. This might be connected with the higher acidity of citrate in relation to acetate or phosphate.

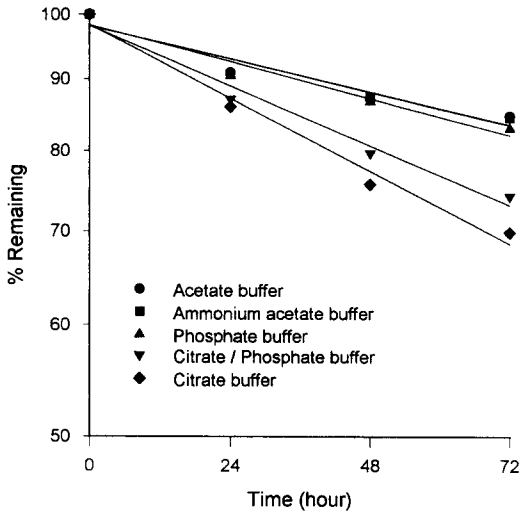

Fig. 4. First-order plots for the degradation of GHRP-6 in various buffer species at pH 5.5 and 80 ± 0.5°C.

Table 2
Effect of buffer concentration on the degradation of GHRP-6 in pH 5.5 at 80 ± 0.5°C

Buffer solutions	% GHRP-6 remaining
0.01 M citrate/0.02 M citric acid	69.90 ± 2.51
0.1 M citrate/0.2 M citric acid	36.45 ± 3.44
0.01 M citric acid/0.02 M phosphate	73.52 ± 2.62
0.1 M citric acid/0.2 M phosphate	56.14 ± 2.57
0.01 M acetate/0.02 M acetic acid	85.05 ± 1.87
0.1 M acetate/0.2 M acetic acid	82.39 ± 2.09
0.01 M ammonium acetate/0.02 M acetic acid	84.54 ± 1.69
0.1 M ammonium acetate/0.2 M acetic acid	82.38 ± 2.60
0.01 M phosphate/0.02 M phosphoric acid	83.02 ± 2.46
0.1 M phosphate/0.2 M phosphoric acid	78.78 ± 3.11

Variation of ionic strength adjusted with potassium chloride had a negligible effect on the degradation of GHRP-6 in acetate buffer solution at 80°C, as shown in Table 3.

Arrhenius plots of the data observed in kinetic studies at four different temperatures (40–90°C) in acetate buffer at pH 5.5 were reasonably linear, with a correlation coefficient greater than 0.99, as shown in Fig. 5. The slope gives an activation energy of the overall reaction of 22.21 kcal/mol for GHRP-6. Extrapolation to 20°C gives a pre-

Table 3
Effect of ionic strength adjusted with potassium chloride on the degradation of GHRP-6 in pH 5.5 acetate buffer at 80 ± 0.5°C

Amount of potassium chloride	% GHRP-6 remaining
0.00 M	85.05 ± 1.87
0.01 M	82.34 ± 2.88
0.02 M	82.87 ± 2.40
0.05 M	82.80 ± 2.97
0.10 M	82.26 ± 3.08
0.20 M	81.58 ± 3.36
0.50 M	81.07 ± 2.65

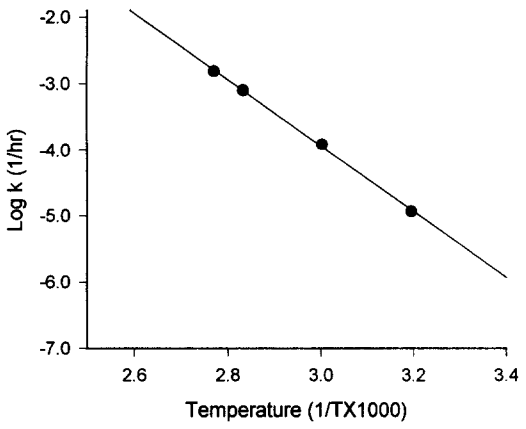

Fig. 5. Arrhenius plot of log rate constant vs. $1/T$ for the degradation of GHRP-6 in pH 5.5 acetate buffer.

dicted mean $t_{90\%}$ at pH 5.5 of 4.73 years (95% confidence) for GHRP-6.

In conclusion, the degradation chromatograms of GHRP-6 were confirmed in different pH solutions, and the observed degradation reaction rates approximately followed first-order kinetics with respect to GHRP-6. Different buffer species as well as their concentrations had different effects on GHRP-6 stability, while the maximum stability of GHRP-6 was revealed to be in acetate buffer of pH 5.5–6.0.

References

Bercu, B.B., Weideman, C.A. and Walker, R.F., Sex differences in growth hormone secretion by rats administered GH-releasing hexapeptide. *Endocrinology*, 129 (1991) 2592–2598.

Bercu, B.B., Yang, S.W., Msuda, R. and Walker, R.F., Role of selected endogenous peptides in growth hormone-releasing hexapeptide activity: analysis of growth hormone-releasing hormone, thyroid hormone-releasing hormone, and gonadotropin-releasing hormone. *Endocrinology*, 130 (1992) 2579–2586.

Bowers, C.Y., Momany, F.A., Reynolds, G.A. and Hong, A., On the in vitro and in vivo activity of a new synthetic hexapeptide that acts on the pituitary to specifically release growth hormone. *Endocrinology*, 114 (1984) 1537–1545.

Bowers, C.Y., Sartor, A.O., Reynold, G.A. and Badger, T.M., On the actions of the growth hormone-releasing hexapeptide GHRP. *Endocrinology*, 128 (1991) 2028–2035.

Bowers, C.Y., Alster, D.K. and Frentz, J.M., The growth hormone-releasing activity of a synthetic hexapeptide in normal men and short statured children after oral administration. *J. Clin. Endocrinol. Metab.*, 74 (1992) 292–298.

Fairhall, K.M., Mynett, A. and Robinson, I.C.A.F., Central effects of growth hormone-releasing hexapeptide (GHRP-6) on growth hormone release are inhibited by central somatostatin action. *J. Endocrinol.*, 144 (1995) 555–560.

Fletcher, T.P., Thomas, G.B., Willoughby, J.O. and Clarke, I.J., Constitutive growth hormone secretion in sheep after hypothalamopituitary disconnection and the direct in vivo pituitary effect of growth hormone releasing peptide 6. *Neuroendocrinology*, 60 (1994) 76–86.

Hartman, M.L., Farello, G., Pezzoli, S.S. and Thorner, M.O., Oral administration of growth hormone (GH)-releasing peptide stimulates GH secretion in normal men. *J. Clin. Endocrinol. Metab.*, 74 (1992) 1378–1384.

Lee, K.C., Lee, Y.J., Song, H.M., Chun, C.J. and DeLuca, P.P., Degradation of synthetic salmon calcitonin in aqueous solution. *Pharm. Res.*, 9 (1992) 1521–1523.

Manning, M.C., Patel, K. and Borchardt, R.T., Stability of protein pharmaceuticals. *Pharm. Res.*, 6 (1989) 903–918.

Nelson, A.H., Walker, R.F., Codd, E.E. and Barone, F.C., Intranasal activity of the growth hormone releasing peptide His-D-Trp-Ala-Trp-D-Phe-Lys-NH_2 in conscious dogs. *Life Sci.*, 48 (1991) 2283–2288.

Oh, C.K., Ryan, F.M., Christensen, G.A., Smith, P.L. and Gochoco, C.H., Barriers to the transport of growth hormone releasing peptide across epithelial membranes. *Pharm. Res.*, 8 (1991) S160.

Oyler, A.R., Naldi, R.E., Lloyd, J.R., Graden, D.A., Shaw, C.J. and Cotter, M.L., Characterization of the solution degradation products of histrelin, a gonadotropin releasing hormone agonist. *J. Pharm. Sci.*, 80 (1991) 271–275.

Popovic, V., Damjanovic, S., Micic, D., Djurovic, M., Dieguez, C. and Casanueva, F.F., Blocked growth hormone-releasing peptide (GHRP-6)-induced GH secretion and absence of the synergic action of GHRP-6 plus GH-releasing hormone in patients with hypothalamopituitary disconnection: evidence that GHRP-6 main action is exerted at the hypothalamic level. *J. Clin. Endocrinol. Metab.*, 80 (1995) 942–947.

Reardon, P.M., Gochoco, C.H., Audus, K.L., Wilson, G. and Smith, P.L., In vitro nasal transport across ovine mucosa: effects of ammonium glycyrrhizinate on electrical properties and permeability of growth hormone releasing peptide, mannitol, and lucifer yellow. *Pharm. Res.*, 10 (1993) 553–561.

Renner, U., Brockmeier, S., Strasburger, C.J., Lange, M., Schopohl, J., Muller, O.A., Werder, K.V. and Stalla, G.K., Growth hormone (GH)-releasing peptide stimulation of GH release from human somatotroph adenoma cells: interaction with GH-releasing hormone, thyrotropin-releasing hormone, and octreotide. *J. Clin. Endocrinol. Metab.*, 78 (1994) 1090–1096.

Samanen, H.M., Physical biochemistry of peptide drugs: structure, properties and stabilities of peptides compared with proteins. In Lee, V.H.L. (Ed), *Peptide and Protein Drug Delivery*, Marcel Dekker, New York, 1991, pp. 137–166.

Vora, J.K., Christensen, G.A., Reginato, M.J., Macia, R.A., Gochoco, C.H. and Oh, C.K., Influence of formulation variables on plasma growth hormone levels following intranasal administration of growth hormone-releasing peptide in anesthetized rats. *J. Controlled Release*, 24 (1993) 193–200.

Walker, R.F., Codd, E.E., Barone, F.C., Nelson, A.H., Goodwin, T. and Campbell, S.A., Oral activity of the growth hormone releasing peptide His-D-Trp-Ala-Trp-D-Phe-Lys-NH_2 in rats, dogs and monkeys. *Life Sci.*, 47 (1990) 29–36.